5. Vaccine development: basic considerations

Asexual blood stage vaccines: from merozoites to peptides

L. H. Perrin, Ph. Simitsek and P. Fasani-Ghersa *Division of Haematology, Geneva University Hospital, 25 rue Micheli-du-Crest, 1211 Geneva 4, Switzerland*

Abstract

Asexual blood stage proliferation is responsible for the morbidity and mortality associated with malaria infection in man. These developmental stages are therefore obvious targets for the development of malaria vaccines. Several asexual blood stage components have been identified as potential candidates for the development of vaccines and some of them have been shown, following immunization, to induce at least partial protection in a variety of *Plasmodium*-host combinations. Studies on defined parasite components and on synthetic peptides derived from them have revealed new insights at the molecular level into parasite mechanisms involved in propagation and survival in the infected host, and into the interaction between parasite components and the host immune system. Practical application of these findings is likely to provide the basis for the design of more appropriate antigens for the development of vaccines.

Introduction

Epidemiological studies have shown that the development of resistance to malaria in man depends on both the degree and the duration of exposure to the parasite. A primary malaria infection is not followed by the development of solid immunity and most of the deaths occur in young children in endemic areas and in infected adults without previous exposure to malaria. Immunity builds up in the course of successive infections and sterile immunity is probably never achieved. In endemic areas clinically significant malaria becomes infrequent in adults with the exception of pregnant women; however transient parasitaemia is still observed.

Asexual blood stage proliferation is responsible for all the morbidity and mortality associated with malaria in man and is the obvious target for the development of malaria vaccines. In practical terms one may consider two kinds of asexual blood stage vaccines: first, a fully efficient vaccine able to confer sterile immunity; such a vaccine has to do better than nature and represents a difficult challenge: second, a vaccine capable of stimulating a degree of protection which would enable the vaccinated individuals to overcome subsequent infection without suffering major illness. In endemic areas this kind of vaccine would transform the immune system of an unprotected child into that of an adult having experienced multiple malaria infections.

Identification of antigens involved in protection; experimental immunization

Immunization experiments have been carried out with merozoites, schizonts, or both in a variety of host-*Plasmodium* combinations, including monkeys immunized with *P. falciparum*. These experiments established that at least partial protection can be induced artificially (SIDDIQUI, 1977). Another drawback of immunization experiments with sporozoites, asexual blood stages and gametes was the finding that immunity induced by these vaccines was stage specific. Immunity to sporozoites is without effect on parasitaemia, which may develop if some sporozoites evade immunity, and asexual blood stage vaccine did not prevent the development of exoerythrocytic schizogony in the liver. The production of whole merozoites or schizonts, or both, in sufficient amounts for large scale immunization programmes is not feasible. In addition these crude preparations contain foreign material such as red blood cell membranes which are likely to induce adverse autoimmune reactions. They may also contain parasite components involved in suppressive responses or elicit diffuse immune responses.

For these reasons subsequent efforts were aimed at the development of subunit vaccines. This implicates as a first step the identification of parasite components which may induce protective responses. This search was greatly facilitated by the development by TRAGER & JENSEN (1976) of an *in vitro* method for the culture of *P. falciparum* erythrocytic stages, which made it possible for a number of laboratories to analyse in detail individual components of both sexual and asexual stages of *P. falciparum* and to conduct biological assays routinely.

Several hundreds of asexual blood stage components are able to raise an immune response but very few of them are helpful for the host. It is therefore a difficult challenge to select the appropriate components and several frequently complementary approaches have been selected, including the following.

(i) Identification of antigens expressed at the surface of merozoites and schizonts by surface labelling.

(ii) Identification of parasite components preferentially recognized by sera of immune adults or sera of protected monkeys.

(iii) Identification of parasite components recognized by monoclonal antibodies Ible to inhibit the growth of asexual blood stages *in vitro*.

(iv) Search for parasite molecules with crucial functions for the development of the parasite or invasion of erythrocytes by merozoites.

(v) Finally, more recently, systematic immunization of experimental monkeys with *P. falciparum* components eluted from gels, determination of the amino acid sequences of components associated with

protection, and biochemical synthesis of corresponding peptides which in turn are used for immunization of monkeys (PATARROYO *et al.*, 1987).

From these investigations a number of potential candidates emerged and we shall now briefly describe some of their characteristics.

Merozoite surface antigens

A protein with a molecular mass in the range of 190–230 kDa has been identified at the surface of schizonts in several malaria species. This protein is processed into a smaller molecule which is the main component exposed at the surface of merozoites. In mice, immunization with this protein purified from *P. yoelii* extract was able to protect the animals from challenge infection (HOLDER & FREEMAN, 1981). The corresponding protein of *P. falciparum* has an apparent molecular mass of 180–200 kDa and is processed into an 83 kDa merozoite surface polypeptide (HOLDER & FREEMAN, 1982). By using a panel of monoclonal antibodies it was shown that this molecule presented a considerable degree of antigenic diversity (MCBRIDE *et al.*, 1985). The gene coding for this protein has been cloned from several *P. falciparum* isolates and can be divided into several blocks ranging in homology from 10 to 87% at the amino acid levels (MCKAY *et al.*, 1985). Several immunization trials have been conducted with this protein in monkeys and have indicated that partial to almost complete protection can be induced by this protein administered with Freund's complete adjuvant (PERRIN *et al.*, 1984; HALL *et al.*, 1984; SIDDIQUI *et al.*, 1987). Immunization with synthetic polypeptides corresponding to the conserved NH_2 part of the molecule and to another area (aa 277–287) also confers partial protection on immunized monkeys (CHEUNG *et al.*, 1986; PATARROYO *et al.*, 1987). Other antigens are also expressed at the surface of the merozoite but they have not been fully characterized.

Antigens associated with the membrane of infected erythrocytes.

Several proteins associated with the membrane of red blood cells containing *P. falciparum* asexual blood stages have been characterized, in particular a ring-infected erythrocyte surface antigen (RESA) and 2 proteins associated with knobs (electron dense protuberances of the plasma membrane of infected erythrocytes): KAHRP (knob-associated histidine-rich protein) and MESA (mature parasite-infected erythrocyte surface antigen).

RESA has a molecular mass of 155 kDa. This antigen is synthesised in trophozoites, accumulates in micronemes (apical structures of merozoites) and, following invasion, becomes associated with the membrane of ring-infected erythrocytes (PERLMANN *et al.*, 1984; BROWN *et al.*, 1985). Antibodies against RESA inhibit the proliferation of asexual blood stages of *P. falciparum in vitro* and it was postulated that these antibodies interfere with the invasion process. The gene coding for RESA has been cloned and it was found that the molecule contained two blocks of repetitive sequences which are the immunodominant epitopes in terms of antibody responses (FAVAROLO *et al.*, 1986; KEMP *et al.*, 1987). Interestingly, antibodies directed against the repeat areas cross-react with at least 6 other asexual blood stage components. Monkeys have been immunized with synthetic peptides corresponding to the repeated sequences and with fusion proteins corresponding to various areas of the molecule. Some groups of monkeys were protected (COLLINS *et al.*, 1986) but the most active area of the molecule is still not identified (R. E. Anders, personal communication).

Erythrocytes containing mature trophozoites and schizonts of *P. falciparum* adhere through the knobs to endothelial cells lining venules in deep tissues. This cytoadherence of parasitized erythrocytes prevents their passage through the spleen and their eventual destruction in this organ. Cytoadherence can be inhibited by antisera in a strain specific manner but an antigenically invariant epitope was also identified at the surface of infected erythrocytes isolated from Gambian patients (MARSH & HOWARD, 1986). Several proteins are present in the knobs and two of them, KAHRP and MESA, have been characterized and part of the gene coding for them has been cloned. These two proteins present a size variation for various *P. falciparum* isolates of 85–105 kDa for KAHRP and 240–300 kDa for MESA. These proteins contain repeated amino acid sequences and differ antigenically among isolates.

Rhoptry proteins.

Rhoptries are apical organelles of merozoites which release their content into erythrocytes at the time of invasion. Monoclonal antibodies directed against a rhoptry component of *P. yoelii* reduce the virulence of infection following passive transfer and monoclonal antibodies directed against 41–82 kDa rhoptry components of *P. falciparum* inhibit the growth of asexual blood stages *in vitro* (PERRIN & DAYAL, 1982). The 82 kDa protein is processed into components of 76 and 65 kDa and is not related to the 41 kDa polypeptide (BRAUN-BRETON *et al.*, 1986). Interestingly the functional activity of both of these components has been clarified: the 82–76 kDa component is a protease and the 41 kDa component displays aldolase activity (U. Certa, personal communication). In addition, immunization of monkeys with either of these peptides induces protective immunity (DUBOIS *et al.*, 1984; PERRIN *et al.*, 1985).

Another rhoptry antigen of *P. falciparum* has been characterized. This protein is located in the peduncle of the rhoptries, is synthesised at the trophozoite stage in the form of a 240 kDa polypeptide which is processed into a 255 kDa polypeptide during schizogony. The 225 kDa protein is recovered quantitatively in the supernatant of culture following merozoite invasion (DUBREMETZ *et al.*, 1988). A third set of rhoptry proteins is composed of three proteins of molecular mass of 105, 130 and 140 kDa which are co-precipitated. These components are unrelated, as shown by protease digestion of individual components and by epitope mapping. The 225 kDa protein and proteins of the 105/130/140 kDa complex have not been evaluated in immunization trials in monkeys.

Other candidates

Several other proteins of asexual blood stages are considered as candidates for the development of asexual blood stage vaccine on the basis of their functional activity or on the basis of successful immunization trials. Several parasite components

have been shown to bind to erythrocyte membranes or to glycophorin A, or both, which may act as binding ligand for merozoites (JUNGERY *et al.*, 1983). These polypeptides are obvious candidates for vaccine development but, like many other *P. falciparum* polypeptides, may present marked antigenic diversity among various isolates. Another potential candidate is the malaria transferrin receptor which has been identified at the surface of erythrocytes. The parasite requires exogenous iron and the receptor internalizes and transports bound ferrotransferrin to the intracellular parasite. The potential of this protein for vaccine development will depend on its divergence from the human receptor (RODRIGUEZ & JUNGERY, 1986). Another potential candidate is a parasitophorous vacuole antigen of *P. falciparum* with a molecular mass of 126–140 kDa. This protein is cleaved upon schizont rupture into 3 major fragments of 50, 47 and 18 kDa which are released into culture medium (DELPLACE *et al.*, 1985). Part of the gene coding for this protein has been cloned and shown to code for amino acid repeats; one of the stretch of repeats being composed of polyserine repeats. Monkeys immunized with the 126–140 kDa protein are protected from a lethal *P. falciparum* challenge (PERRIN *et al.*, 1984).

Synthetic peptides as immunogens

Peptides derived from constant portions of the 83 kDa merozoite surface protein have been used as immunogen after coupling to carrier proteins (CHEUNG *et al.*, 1986; PATARROYO *et al.*, 1987). Similar methods have been applied using polypeptides corresponding to repeats of RESA (COLLING *et al.*, 1986) and to the NH_2 terminal portion of 2 parasite components characterized only by their apparent molecular mass of 55 and 35 kDa (PATARROYO *et al.*, 1987). A partial protection of a lower magnitude than that observed following immunization with the complete corresponding antigens was observed. The antibody titres obtained using synthetic peptides were also relatively low. Finally there is the problem of boosting the response following natural exposure of the immunized individuals. The development of antibodies requires T cell help which was provided in the above immunizations by foreign carriers, which will not be available to induce a booster effect following malaria infection. In this respect T cell activating sites present in the parasite molecules should be present in the vaccine rather than foreign material. In relation to T cell sites, their selection may be a complex process since response to some of them is restricted by the major histocompatibility complex (DEL GIUDICE *et al.*, 1986) and others may be present on areas of the parasite molecules presenting sequence variations among isolates. These elements suggest that the development of malaria vaccines based on asexual blood stage components should rely not only on identification of parasite target antigens and their production but also on the molecular interaction between the parasite components and the host effector mechanisms. In this respect it is still not clear whether the best vaccine should be based on full length parasite components (eventually with excision of limited parts of the molecules involved in negative regulation or containing repeats), these full length proteins having the advantage of containing multiple B and T cell epitopes, or on a cocktail of synthetic peptides with complementary function. The identification of B and T cell epitopes as well as the interaction of the parasite components with the major histocompatibility complex is the subject of numerous promising investigations.

Discussion and Conclusion

It seems appropriate, following the description of candidate antigens for the development of asexual blood stage vaccines, to place this development in the context of *Plasmodium*-host relationships. There is an apparent contradiction between the number of potential targets on malaria parasites for host effector mechanisms and the slow development of protective immunity following experimental immunization. This contradiction is the reflection of the extremely successful adaptation of malaria parasites to the vertebrate hosts. Most of the factors which are involved in parasite survival within the host are well known, such as intracellular development of the parasite, parasite antigenic diversity or variation, or both, polyclonal activation of the host immune system, and immunosuppression, but they have been described until recently from the phenomenological aspect only. Development of *in vitro* culture techniques, monoclonal antibodies, T cell cloning and recombinant DNA technology have provided the tools to analyse these phenomena at the molecular levels on both the parasite and the immune system sides (KEMP *et al.*, 1987; DEL GIUDICE *et al.*, 1986).

Antigenic diversity among various isolates has been studied at the molecular level for several components of asexual blood stage parasites and in particular for the 83 kDa protein which is the major *P. falciparum* merozoite surface component and for parasite components exposed at the surface of erythrocytes containing mature parasites. These components are the subject of intense pressure by the immune system and their high concentraton in the blood of infected individuals increases the opportunity for the selection of mutants. Another characteristic of malaria proteins is the presence of tandemly repeated amino acids which are frequently dominant in term of antibody responses. These repeats may vary or may be deleted (A. Anders & U. Certa, personal communications). It has been postulated that these repeats divert the immune response (the smokescreen model) and prevent the development of truly protective responses (KEMP *et al.*, 1987). In addition they may be involved in polyclonal B and T cell activation and play a role in the immunosuppression associated with malaria. The extent of antigenic diversity observed in malaria can have direct consequences on primary and secondary responses to the infection which require collaboration of B and T cells whose responses can be exquisitely specific for a given epitope. Another mechanism capable of preventing the development of specific protective responses on one hand and on the other hand favouring the development of autoantibodies is the homology of parasite sequences with human proteins. This has been shown, for example, for the region II of the circumsporozoite protein and for the 83 kDa merozoite surface protein (CHEUNG *et al.*, 1986).

All these elements focus our attention on the necessity for both basic and applied research and for a multidisciplinary approach to the development of malaria vaccines.

Acknowledgements

Work in the laboratory of Dr. L. H. Perrin is supported by the UNDP/World Bank/WHO Special Programme in Research and Training in Tropical Diseases, ID no. 870104, and by the Swiss National Research Foundation Grant 3.923.0.87.

References

Braun-Breton, C., Jendoubi, M., Brunet, E., Perrin, L., Scaife, J. & Perreira da Silva, I. (1986). *In vivo* time course of synthesis and processing of major schizont membrane polypeptides in *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **20**, 30–39.

Brown, G. V., Culvenor, I. G., Grewther, P. E., Bianco, A. E., Coppel, R. L., Saint, R. B., Stahl, H. D., Kemp, D. J. & Anders, R. F. (1985). Localisation of the ring infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* in merozoites and ring infected erythrocytes. *Journal of Experimental Medicine*, **162**, 774–779.

Cheung, A., Leban, J., Shaw, A. R., Merkli, B., Stocker, J., Chizzolini, C., Sanders, C. & Perrin, L. H. (1986). Immunisation with synthetic peptides of *Plasmodium falciparum* induces antimerozoite antibodies. *Proceedings of the National Academy of Sciences of the USA*, **83**, 8328–8332.

Collins, W. E., Anders, R. F., Pappaioanou, M., Campbell, G. H., Brown, G. B., Kemps, D. J., Coppel, R. L., Skinner, J. C., Andrysiak, P. M., Favarolo, J. M., Corcoran, L. M., Broderson, J. R., Mitchell, G. F. & Campbell, C. C. (1986). Immunisationof *Aotus* monkeys with recombinant proteins of an erythrocyte surface antigen of *Plasmodium falciparum*. *Nature*, **232**, 259–262.

Del Giudice, G., Cooper, J. A., Merino, J., Verdini, A. S., Pessi, A., Togna, A. R., Engers, H. D., Corradin, G. & Lambert, P. H. (1986). The antibody response in mice to carrier-free synthetic polymers of *Plasmodium falciparum* repetitive epitope is I-A restricted; possible implications for malaria vaccines. *Journal of Immunology*, **137**, 2952–2955.

Delplace, P., Dubremetz, J. F., Fortner, B. & Vernes, A. (1985). A 50 kilo-Dalton exoantigen specific to the merozoite release reinvasion stage of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **17**, 239–245.

Dubois, P., Dedet, J. P., Fandeur, T., Rousilhon, C., Jendoubi, M., Pauillac, S., Mercrereau-Puijalon, O. & Pereira Da Silva, L. (1984). Protective immunization of the squirrel monkey against asexual blood stages of *Plasmodium falciparum* by use of parasite protein fractions. *Proceedings of the National Academy of Sciences of the USA*, **81**, 229–232.

Dubremetz, R. N., Delplace, P., Fortier, B., Tronchin, G. & Vernes, A. (1988). The p225 rhoptry antigen of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **27**, 135–139.

Favarolo, J. M., Coppel, R. L., Corcoran, L. M., Foote, S. J., Brown, G. V., Anders, R. F. & Kemp, D. J. (1986). Structure of the RESA gene of *Plasmodium falciparum*. *Nucleic Acids Research*, **14**, 8265–8277.

Hall, R., Hyde, J. E., Goman, M., Simmons, D. L., Hope, I. A., Mackay, H., Scaife, J., Merkli, B., Richle, R. & Stocker, J. (1984). Major surface antigen of a human malaria parasite cloned and expressed in bacteria. *Nature*, **311**, 379.

Holder, A. A. & Freeman, R. R. (1981). Immunisation against blood stage malaria using purified parasite antigens. *Nature*, **294**, 361–364.

Holder, A. A. & Freeman, R. R. (1982). Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognised by immune serum and monoclonal antibody. *Journal of Experimental Medicine*, **156**, 1528–1538.

Jungery, M., Bolyle, D., Patel, T., Pasvol, G. & Weatherall, D. J. (1983). Lectin like polypeptide of *Plasmodium falciparum* binds to red cell sialoglycoproteins. *Nature*, **301**, 704.

Kemp, D. J., Coppel, R. L. & Andres, R. F. (1987). Repetitive proteins and genes of malaria. *Annual Review of Microbiology*, **41**, 128–142.

Mackay, M., Goman, M., Bone, N., Hyde, J. E., Scaife, J., Certa, U., Stunnenberg, H. & Bujard, H. (1985). Polymorphism of the precursor for the major surface antigens of *Plasmodium falciparum* merozoites: studies at the genetic level. *Embo Journal*, **4**, 3823–3829.

Marsh, K. & Howard, R. J. (1986). Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. *Science*, **231**, 150–153.

McBride, J., Walliker, D. & Morgan, G. (1982). Antigenic diversity in the human malaria parasite, *Plasmodium falciparum*. *Science*, **217**, 254–257.

Patarroyo, M. E., Romero, P., Torres, M. L., Clavijo, P., Moreno, A., Martinez, A., Rodriguez, R., Guzman, F. & Catezas, E. (1987). Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature*, **328**, 629–631.

Perlmann, H., Berzins, K., Wahlgren, M., Carlson, J., Bjorkman, A. & Perlman, P. (1984). Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum*. *Journal of Experimental Medicine*, **159**, 1686–1704.

Perrin, L. & Dayal, R. (1982). Immunity to asexual erythrocytic stages of *Plasmodium falciparum*: role of defined antigens in humoral response. *Immunological Review*, **61**, 245–269.

Perrin, L. H., Merckli, B., Gabra, M. S., Stocker, J., Chizzolini, C. & Richle, R. (1985). Immunisation with a *Plasmodium falciparum* merozoite surface antigen induces a partial immunity in monkeys. *Journal of Clinical Investigation*, **75**, 1718–1724.

Perrin, L. H., Merckli, B., Loche, M., Chizzolini, C., Smart, J. & Richle, R. (1984). Antimalarial immunity in *Saimiri* monkeys. Immunisation with surface components of asexual blood stages. *Journal of Experimental Medicine*, **160**, 441–451.

Rodriguez, M. H. & Jungery, M. (1986). A protein on *Plasmodium falciparum* infected erythrocytes functions as a transferrin receptor. *Nature*, **324**, 388–391.

Siddiqui, W. A. (1977). An effective immunization of experimental monkeys against a human malaria parasite, *Plasmodium falciparum*. *Science*, **197**, 388–389.

Siddiqui, W. A., Tam, L. Q., Kramer, K. J., Hui, G. S. N., Case, S. E., Yamaya, K. M., Chang, S. P., Chan, E. B. T. & Kan, S. C. (1987). Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences of the USA*, **84**, 3014–3018.

Trager, W. & Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science*, **193**, 673–675.